REMARKS

Upon entry of the amendments, claims 14, 16-18, 21, 25-29 will be pending in the application.

Applicants provide the followings comments to the Office Action of September 23, 2003.

Election

Applicants confirm that election of Group III. Applicants have canceled the non-elected claims and reserve the right to file divisional applications at a later time.

Priority

With this Amendment, Applicants have concurrently filed verified English translations of the priority document (DE 10043332.4) filed on September 2, 2000 and the priority document (DE 10033426.5) filed on July 10, 2001.

Objections to the Specification

Applicants have amended the title as suggested by the Examiner. Applicants have also amended the Abstract to address the objections raised in the Office Action. Applicants also respectfully assert that sigC has a known function as discussed in the anticipation rejection section. Reference is made to the enclosed Swiss-Prot:Q03066 and 7083, which reference sigC.

Objections to the Claims

Objections were raised to claims 22, 23, and 27. Claims 22 and 23 have been canceled. Claim 27 has been amended to more clearly identify the coryneform bacteria.

Claim Rejections – 35 U.S.C. § 112 (second paragraph)

Claims 14-27 and 29 are rejected as being indefinite.

In response, Applicants have amended the claims to address the Examiner's rejection. Applicants respectfully submit that the amended claims comply with the second paragraph of 35 U.S.C. § 112.

Claim Rejections – 35 U.S.C. § 112 (first paragraph)

Claims 14-27 and 29 have been rejected for failing to meet the written description requirement.

In response, Applicants have amended the claims to address the Examiner's concerns. Applicants respectfully submit that the claims comply with the written description requirement of 35 U.S.C. § 112.

Claim Rejections – 35 U.S.C. § 102

The Office Action sets forth the following anticipation rejections:

1) claims 14-18, 21-23, and 27 are rejected under 35 U.S.C. §102(b) based on Kimura (EP 0864654); and

2) claims 14-18, 21-23, and 27 are rejected based on Nakagawa (EP 1108790).

Applicants have carefully considered the rejections set forth in the Office Action, but respectfully request that they be withdrawn.

Regarding the first rejection, Applicants note that Kimura discloses transformed organisms with a gene of E. coli coding for a heat shock protein and a gene coding for a factor which specifically functions for the heat shock protein to enhance expression amount of the heat shock protein in cells. Applicants have enclosed an excerpt from *A Short Course in*

Bacterial Genetics, which explains that rpoH is a regulatory gene for proteins induced at high temperatures.

In contrast, it is known in the art that sigC is a gene associated with RNA polymerase sigma-C factor, which is an initiation factor that promotes attachment of the RNA polymerase to specific initiation sites and then is released. This sigma factor is essential for normal fruiting body formation. Reference is made to the enclosed Swiss-Prot documents.

Applicants believe that Kimura lacks any inherent or explicit disclosure regarding sigma-C factor being overexpressed to enhance the production of L-amino acids by bacteria. Kimura fails to disclose "fermenting coryneform bacteria which produce a desired L-amino acid comprising an overexpressed polynucleotide sigC wherein said polynucleotide comprises a nucleotide sequence of SEQ ID NO:1 and encodes a polypeptide having an RNA polymerase sigma-C factor activity". As such, the anticipation rejection should be withdrawn.

Regarding the second rejection, Applicants assert that Nakagawa (published on June 20, 2001) fails to qualify as prior art based upon the perfected filing date of the present application. Furthermore, Applicants believe that Nakagawa lacks disclosure regarding fermenting l-amino acid producing bacteria that overexpress sigC. Therefore, the second rejection should be withdrawn.

CONCLUSION

Applicants request allowance of the application. If any additional fees are due in connection with the filing of this response, please charge the fees to Deposit Account No. 02-4300. Any overpayment can be credited to Deposit Account No. 02-4300.

Respectfully submitted,

SMITH, GAMBRELL & RUSSELL, LLP

Date : December 23, 2003

Brandon Boss, Reg. No. 46,567*
1850 M Street, NW – Suite 800
Washington, DC 20036
Telephone : 202/263-4300
Facsimile : 202/263-4329

* Practice is limited to matters and proceeding before federal courts and agencies.

A SHORT COURSE IN BACTERIAL GENETICS

A Laboratory Manual and Handbook
for *Escherichia coli* and Related Bacteria

Printed in the United States of America

9 8 7 6 5 4 3 2 1

Text design by Emily Harste
Cover design by Leon Bolognesi

Front cover: This strain of *Escherichia coli* with an altered *lacZ* gene reverts to Lac^+ only when certain transversion mutations occur. When grown on solid media containing a mix of β-galactosides, such mutants appear as blue Lac^+ papillae, or miniature colonies, growing out of a larger white colony of cells containing the still unreverted *lacZ* gene. (Photograph by J.H. Miller and his colleagues, University of California, Los Angeles.)

Back cover: Heterozygous colonies stained for the arabinose constitutive phenotype (*araC*) by the method of Lin et al. (*Biochim. Biophys. Acta*, vol. 60, pp. 422–424 [1962]). Stain: 1% solution of 2,3,5-triphenyltetrazolium chloride.

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Table 1 *Continued*

Gene symbol	Mnemonic	Map position (min)[a]	Alternate gene symbols; phenotypic trait affected[b]	Reference(s)[c]
rimI	Ribosomal modification	99	Modification of 30S ribosomal subunit protein S18; acetylation of N-terminal alanine	B, C, 1209
rimJ	Ribosomal modification	(32)	Modification of 30S ribosomal subunit protein S5; acetylation of N-terminal alanine	B, 524, 1209
rimL	Ribosomal modification	(33)	Modification of 30S ribosomal subunit protein L7; acetylation of N-terminal serine	C
rit		89	Affects thermolability of 50S ribosomal subunit	B
rlpA		15	A minor lipoprotein	1076
rlpB		15	A minor lipoprotein	1076
rna	RNase	14	*rns*, *rnsA*; RNase I	A
rnb	RNase	29	RNase II	B, C
rnc	RNase	55	RNase III	A, B, 699, 787, 1160
rnd	RNase	40	RNase D	C, 1216
rne	RNase	24	RNase E activity	B, C, 893
rnh	RNase	5	*dasF*, *herA*, *sdrA*, *sin*; RNase H (EC 3.1.26.4)	C, 225, 550, 690, 771, 806, 813, 1108
rnpA	RNase	83	RNase P, protein component	435
rnpB	RNase	70	RNase P, RNA subunit, M1 RNA	B, C, 758, 895, 896, 952
rodA		15	See *mrdB*	
rpiA		63	Ribose phosphate isomerase (EC 5.3.1.6), constitutive	A
rplA	Ribosomal protein, large	90	50S ribosomal subunit protein L1	A, B, C, 283, 491, 889
rplB	Ribosomal protein, large	73	50S ribosomal subunit protein L2	A, B, 1222
rplC	Ribosomal protein, large	73	50S ribosomal subunit protein L3	A, B, 1222
rplD	Ribosomal protein, large	73	*eryA*; 50S ribosomal subunit protein L4	A, B, C, 1222
rplE	Ribosomal protein, large	73	50S ribosomal subunit protein L5	A, B, 181
rplF	Ribosomal protein, large	73	50S ribosomal subunit protein L6	A, B, 181
rplI	Ribosomal protein, large	96	50S ribosomal subunit protein L9	B, 974
rplJ	Ribosomal protein, large	90	50S ribosomal subunit protein L10	A, B, C, 203, 283, 491, 889
rplK	Ribosomal protein, large	90	*relC*; 50S ribosomal subunit protein L11	A, B, C, 283, 491, 889
rplL	Ribosomal protein, large	90	50S ribosomal subunit protein L7/L12	A, B, C, 283, 491, 889
rplM	Ribosomal protein, large	70	50S ribosomal subunit protein L13	C, 514
rplN	Ribosomal protein, large	73	50S ribosomal subunit protein L14	A, B, 181
rplO	Ribosomal protein, large	73	50S ribosomal subunit protein L15	A, B, 181, 516
rplP	Ribosomal protein, large	73	50S ribosomal subunit protein L16	A, B, 1222
rplQ	Ribosomal protein, large	73	50S ribosomal subunit protein L17	A, B, C, 77, 181, 730
rplR	Ribosomal protein, large	73	50S ribosomal subunit protein L18	A, B, 181
rplS	Ribosomal protein, large	57	50S ribosomal subunit protein L19	B, 164
rplT	Ribosomal protein, large	38	*pdzA*; 50S ribosomal subunit protein L20	320
rplU	Ribosomal protein, large	69	50S ribosomal subunit protein L21	B
rplV	Ribosomal protein, large	73	50S ribosomal subunit protein L22	A, B, 1222
rplW	Ribosomal protein, large	73	50S ribosomal subunit protein L23	B, 1222
rplX	Ribosomal protein, large	73	50S ribosomal subunit protein L24	A, B, 181, 235
rplY	Ribosomal protein, large	48	50S ribosomal subunit protein L25	B
rpmA	Ribosomal protein, large	69	50S ribosomal subunit protein L27	B
rpmB	Ribosomal protein, large	82	50S ribosomal subunit protein L28	B, C, 1031
rpmC	Ribosomal protein, large	73	50S ribosomal subunit protein L29	A, B, 1222
rpmD	Ribosomal protein, large	73	50S ribosomal subunit protein L30	A, B, 181
rpmE	Ribosomal protein, large	89	50S ribosomal subunit protein L31	C
rpmF	Ribosomal protein, large	24	50S ribosomal subunit protein L32	524
rpmG	Ribosomal protein, large	82	50S ribosomal subunit protein L33	B, C, 1031
rpmH	Ribosomal protein, large	83	*rimA*, *ssaF*; 50S ribosomal subunit protein L34	C, 823, O
rpmI	Ribosomal protein, large	38	50S ribosomal subunit protein A	1149
rpmJ	Ribosomal protein, large	73	50S ribosomal subunit protein X	181, 1149
rpoA	RNA polymerase	73	RNA polymerase (EC 2.7.7.6), α subunit	A, B, C, 77, 181, 730, 928, 929
rpoB	RNA polymerase	90	*groN*, *nitB*, *rif*, *ron*, *stl*, *stv*, *tabD*; RNA polymerase (EC 2.7.7.6), β subunit	A, B, C, 283, 491, 889
rpoC	RNA polymerase	90	*tabD*; RNA polymerase (EC 2.7.7.6), β subunit	A, B, C, 283, 889
rpoD	RNA polymerase	67	*alt*; RNA polymerase (EC 2.7.7.6), σ^{70} subunit	B, C, 157, 677, 1086
rpoH	RNA polymerase	76	*fam*, *hin*, *htpR*; RNA polymerase (EC 2.7.7.6), σ^{32} subunit; regulatory gene for proteins induced at high temperatures	C, 416, 417, 614, 792, 1098, 1116, 1213
rpoN	RNA polymerase	70	*glnF*, *ntrA*; RNA polymerase (EC 2.7.7.6), σ^{60} subunit	B, C, 70, 177, 467, 494
rpsA	Ribosomal protein, small	21	*ssyF*; 30S ribosomal subunit protein S1	B, C, 291, 841, 1001
rpsB	Ribosomal protein, small	4	30S ribosomal subunit protein S2	A, B, C

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[Entry info] [Name and origin] [References] [Comments] [Cross-references] [Keywords] [Features] [Sequence] [Tools]

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Entry information

Entry name	**RPSC_MYXXA**
Primary accession number	**Q07083**
Secondary accession numbers	None
Entered in Swiss-Prot in	Release 30, October 1994
Sequence was last modified in	Release 30, October 1994
Annotations were last modified in	Release 31, February 1995

Name and origin of the protein

Protein name	**RNA polymerase sigma-C factor**
Synonyms	None
Gene name	**SIGC**
From	Myxococcus xanthus [TaxID: 34]
Taxonomy	Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales; Cystobacterineae; Myxococcaceae; Myxococcus.

References

[1] SEQUENCE FROM NUCLEIC ACID.
STRAIN=FB / DZF1;
MEDLINE=93273699; PubMed=8501037; [NCBI, ExPASy, EBI, Israel, Japan]
Apelian D., Inouye S.;
"A new putative sigma factor of Myxococcus xanthus.";
J. Bacteriol. 175:3335-3342(1993).

Comments

- ***FUNCTION***: THE SIGMA FACTOR IS AN INITIATION FACTOR THAT PROMOTES ATTACHMENT OF THE RNA POLYMERASE TO SPECIFIC INITIATION SITES AND THEN IS RELEASED. THIS SIGMA FACTOR IS ESSENTIAL FOR NORMAL FRUITING BODY FORMATION.
- ***SIMILARITY***: Belongs to the sigma-70 factor family.

Copyright

Cross-references

EMBL	L12992; AAA25408.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence]
PIR	A40587; A40587.
HSSP	P00579; 1SIG. [HSSP ENTRY / PDB]
InterPro	IPR009043; RNA_pol_sigma. IPR009042; Sigma70_r1_2. IPR007627; Sigma70_r2. IPR007624; Sigma70_r3. IPR007630; Sigma70_r4. IPR000943; Sigma_70. Graphical view of domain structure.
Pfam	PF00140; sigma70_r1_2; 1. PF04542; sigma70_r2; 1. PF04539; sigma70_r3; 1. PF04545; sigma70_r4; 1.
PRINTS	PR00046; SIGMA70FCT.
PROSITE	PS00715; SIGMA70_1; FALSE_NEG. PS00716; SIGMA70_2; FALSE_NEG.
ProDom	[Domain structure / List of seq. sharing at least 1 domain]
HOBACGEN	[Family / Alignment / Tree]
BLOCKS	Q07083.
ProtoNet	Q07083.
ProtoMap	Q07083.
PRESAGE	Q07083.
DIP	Q07083.
ModBase	Q07083.
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Keywords

Transcription regulation; **Sigma factor**; **DNA-directed RNA polymerase**; **DNA-binding**.

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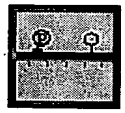
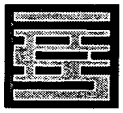
 Feature table viewer

 Feature aligner

Key	From	To	Length	Description
DOMAIN	73	86	14	POLYMERASE CORE BINDING (*POTENTIAL*).
DNA_BIND	250	269	20	H-T-H MOTIF (*BY SIMILARITY*).

Sequence information

Length: **295 AA** Molecular weight: **33433 Da** CRC64: **DB2D4E7832C7FA49** [This is a checksum on the sequence]

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MQASNSFSSP DSLSTYLSEI NQYPLLTQPQ EQELSKRFRA GDLAAGHQLV TANLRFVVKV

        70         80         90        100        110        120
         |          |          |          |          |          |
AYEYRSYGLK MSDLIQEANI GLMKAVQKFD PDKGIRLISY AVWWIRAYIQ NCILKNWSLV

       130        140        150        160        170        180
         |          |          |          |          |          |
KLGTTQAQRR LFFSLARTRR ELEKMGAGDA NVVNAEEIAR KLNVKASEVR EMEQRMGGRD
```

```
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        |           |           |           |           |           |
LSLDAPMGED GDATHLDFVE SESVSAVDEV ADRQQANLTR ELVQRALRRL DPRERFIIEQ

       250         260         270         280         290
        |           |           |           |           |
RVMGDAEMTL SELGEHFGFS RERARQLEIR AKDKLKLALV TLMAEAGVDE STLNA
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Sequence analysis tools: ProtParam, ProtScale, Compute pI/Mw, PeptideMass, PeptideCutter, Dotlet (Java)

ScanProsite, MotifScan

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Entry information

Entry name	**RPSC_ANASP**
Primary accession number	**Q03066**
Secondary accession numbers	None
Entered in Swiss-Prot in	Release 28, February 1994
Sequence was last modified in	Release 41, February 2003
Annotations were last modified in	Release 41, February 2003

Name and origin of the protein

Protein name	**RNA polymerase sigma-C factor**
Synonyms	None
Gene name	**SIGC** or **ALL1692**
From	Anabaena sp. (strain PCC 7120) [TaxID: 103690]
Taxonomy	Bacteria; Cyanobacteria; Nostocales; Nostocaceae; Nostoc.

References

[1] SEQUENCE FROM NUCLEIC ACID.
MEDLINE=93054341; PubMed=1385387; [NCBI, ExPASy, EBI, Israel, Japan]
Brahamsha B., Haselkorn R.;
"Identification of multiple RNA polymerase sigma factor homologs in the cyanobacterium Anabaena sp. strain PCC 7120: cloning, expression, and inactivation of the sigB and sigC genes.";
J. Bacteriol. 174:7273-7282(1992).

[2] SEQUENCE FROM NUCLEIC ACID.
MEDLINE=21595285; PubMed=11759840; [NCBI, ExPASy, EBI, Israel, Japan]
Kaneko T., Nakamura Y., Wolk C.P., Kuritz T., Sasamoto S., Watanabe A., Iriguchi M., Ishikawa A., Kawashima K., Kimura T., Kishida Y., Kohara M., Matsumoto M., Matsuno A., Muraki A., Nakazaki N., Shimpo S., Sugimoto M., Takazawa M., Yamada M., Yasuda M., Tabata S.;
"Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium Anabaena sp. strain PCC 7120.";
DNA Res. 8:205-213(2001).

Comments

- ***FUNCTION***: The sigma factor is an initiation factor that promotes attachment of the RNA

polymerase to specific initiation sites and then is released.
- ***SIMILARITY***: Belongs to the sigma-70 factor family.

Copyright

Cross-references

EMBL	M95759; AAA22047.1; -. [EMBL / GenBank / DDBJ] [CoDingSequence] AP003586; BAB78058.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence]
PIR	AF2017; AF2017. C47017; C47017.
HSSP	P00579; 1SIG. [HSSP ENTRY / PDB]
CMR	Q03066; ALL1692.
InterPro	IPR009043; RNA_pol_sigma. IPR009042; Sigma70_r1_2. IPR007627; Sigma70_r2. IPR007624; Sigma70_r3. IPR007630; Sigma70_r4. IPR000943; Sigma_70. Graphical view of domain structure.
Pfam	PF00140; sigma70_r1_2; 1. PF04542; sigma70_r2; 1. PF04539; sigma70_r3; 1. PF04545; sigma70_r4; 1.
PRINTS	PR00046; SIGMA70FCT.
PROSITE	PS00715; SIGMA70_1; 1. PS00716; SIGMA70_2; 1.
ProDom	[Domain structure / List of seq. sharing at least 1 domain]
HOBACGEN	[Family / Alignment / Tree]
BLOCKS	Q03066.
ProtoNet	Q03066.
ProtoMap	Q03066.
PRESAGE	Q03066.
DIP	Q03066.
ModBase	Q03066.
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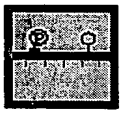
Keywords

Transcription regulation; **Sigma factor**; **DNA-directed RNA polymerase**; **DNA-binding**; **Complete proteome**.

Features

 Feature table viewer

 Feature aligner

Key	From	To	Length	Description
DOMAIN	205	218	14	POLYMERASE CORE BINDING (*POTENTIAL*).
DNA_BIND	374	393	20	H-T-H MOTIF (*BY SIMILARITY*).
CONFLICT	52	52		A -> R (IN REF. 1).

Sequence information

Length: **416 AA** Molecular weight: **47374 Da** CRC64: **0467340EA278964F** [This is a checksum on the sequence]

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MPATSFYADA AYNTQKSRQA LDPDIAIDDS DLSVDEIQEL EIAAADPATF GASANRRSTD

        70         80         90        100        110        120
         |          |          |          |          |          |
LVRLYLQEIG RVRLLGRDEE VSEAQKVQRY LKLRIVLANA VKQGDEVATP YLHLIEVQER

       130        140        150        160        170        180
         |          |          |          |          |          |
LASELGHRPS LERWAATAGI NLCDLKPILS EGKRRWAEIA KMTVEELEKM QSQGLQSKEH

       190        200        210        220        230        240
         |          |          |          |          |          |
MIKANLRLVV SVAKKYQNRG LELLDLVQEG TLGLERAVEK FDPTKGYRFS TYAYWWIRQG

       250        260        270        280        290        300
         |          |          |          |          |          |
ITRAIATSSR TIRLPVHITE KLNKIKKAQR KIAQEKGRTP TLEDLAIELD MTPTQVREVL

       310        320        330        340        350        360
         |          |          |          |          |          |
LRVPRSVSLE TKVGKDKDTE LGELLETDGV TPEEMLMRES LQRDLQHLLA DLTSRERDVI

       370        380        390        400        410
         |          |          |          |          |
LMRFGLADGH PYSLAEIGRA LDLSRERVRQ IESKALQKLR QPKRRNLIRD YLESLS
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BLAST BLAST submission on ExPASy/SIB or at NCBI (USA)

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ScanProsite, MotifScan

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION

I, John F. Moloney, BSc., MIL., CChem., MRSC., translator to Taylor and Meyer of 20 Kingsmead Road, London SW2 3JD, England, do solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and German languages;
2. That the following is a true translation made by me into the English language of German Priority Text Application No. 101 33 426.5;
3. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true;
   and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

Signed, this 23rd day of October 2003,

J. F. Moloney

Eynsham, Oxon OX29 4QD, England

FEDERAL REPUBLIC OF GERMANY

Certificate of Priority for Filing of a Patent Application

Filing number:	101 33 426.5
Filing date:	10th July 2001
Applicant/Proprietor:	Degussa AG, Düsseldorf/Germany
Title:	Nucleotide sequences coding for the sigC gene
Priority:	02.09.2000 DE 100 43 332.4
IPC:	C 12 N, C 07 H, C 12 Q

The attached papers are a true and accurate reproduction of the original documents for this patent application.

Munich, 27th September 2001

On behalf of the President of the German Patent and Trade Mark Office

(signature)

Ebert

Nucleotide Sequences Coding for the sigC gene

The subject of the present invention are nucleotide sequences of coryneform bacteria coding for the sigC gene and a process for the enzymatic production of amino acids using bacteria in which the sigC gene is enhanced.

Prior Art

L-amino acids are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry and, most especially, in animal nutrition.

It is known that amino acids can be produced by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. On account of the great importance of amino acids efforts are constantly being made to improve the production processes. Process improvements may involve fermentation technology measures such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form by for example ion exchange chromatography or the intrinsic performance properties of the microorganism itself.

In order to improve the performance properties of these microorganisms methods involving mutagenesis, selection and mutant selection are employed. In this way strains are obtained that are resistant to antimetabolites or are auxotrophic for regulatorily important metabolites, and that produce amino acids.

For some years methods of recombinant DNA technology have also been used to improve L-amino acid-producing strains of

corynebacterium, by amplifying individual amino acid biosynthesis genes and investigating the effect on amino acid production.

Object of the Invention

The inventors have been involved in providing new techniques for the improved enzymatic production of amino acids.

Description of the Invention

When L-amino acids or amino acids are mentioned hereinafter, it is understood that this refers to one or more amino acids including their salts, selected from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. Lysine is particularly preferred.

The present invention provides an isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence coding for the sigC gene, selected from the group

a) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide that contains the amino acid sequence of SEQ ID No. 2,

b) polynucleotide coding for a polypeptide that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide that is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of the sigma factor C.

The present invention also provides the aforementioned polynucleotide, which is preferably a replicable DNA containing:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence that corresponds to the sequence (i) within the region of degeneracy of the genetic code, or

(iii) at least one sequence that hybridises with the sequence that is complementary to the sequence (i) or (ii), and optionally

(iv) functionally neutral sense mutations in (i).

The invention furthermore provides

a replicable polynucleotide, in particular DNA, containing the nucleotide sequence as shown in SEQ ID No. 1;

a polynucleotide coding for a polypeptide that contains the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

coryneform bacteria that contain the vector or in which the sigC gene is enhanced.

The present invention moreover provides polynucleotides that consist substantially of a polynucleotide sequence that can be obtained by screening by means of hybridisation of a corresponding gene library of a coryneform bacterium that contains the complete gene or parts thereof, with a probe that contains the sequence of the polynucleotide of the invention according to SEQ ID No. 1 or a fragment thereof, and isolation of the aforementioned polynucleotide sequence.

Polynucleotides that contain the sequences according to the invention are suitable as hybridisation probes for RNA, cDNA and DNA in order to isolate nucleic acids or polynucleotides or genes in their full length that code for the sigma factor C, or to isolate such nucleic acids or polynucleotides or genes that have a high sequence similarity to that of the sigC genes. They are also suitable for incorporation in so-called "arrays", "micro arrays" or "DNA chips" in order to detect and determine the corresponding polynucleotides.

Polynucleotides that contain the sequences according to the invention are furthermore suitable as primers with the aid of which, and by employing the polymerase chain reaction (PCR), DNA of genes can be produced that code for the sigma factor C.

Such oligonucleotides serving as probes or primers contain at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, and most particularly preferably at least 15, 16, 17, 18 or 19 successive nucleotides. Also suitable are oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40, or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides. Also suitable if

necessary are oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides.

"Isolated" denotes separated from its natural environment.

"Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, which may be unmodified RNA or DNA or modified RNA or DNA.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment produced therefrom, and also polynucleotides that are at least 70% to 80%, preferably at least 81% to 85%, and particularly preferably at least 86% to 90%, and most particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment produced therefrom.

The term "polypeptides" is understood to mean peptides or proteins that contain two or more amino acids bound by peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of the sigma factor C and also those that are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90%, and most particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2 and that have the aforementioned activity.

The invention furthermore provides a process for the enzymatic production of amino acids selected from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-

isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine, using coryneform bacteria that in particular already produce amino acids and in which the nucleotide sequences coding for the sigC gene are enhanced, in particular overexpressed.

The term "enhancement" describes in this connection the raising of the intracellular activity of one or more enzymes in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, using a strong promoter, or using a gene that codes for a corresponding enzyme having a high activity, and optionally combining these measures.

By enhancement measures, in particular overexpression, the activity or concentration of the corresponding protein is in general raised by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most up to 1000% or 2000%, referred to the wild type protein and/or to the activity or concentration of the protein in the starting microorganism.

The microorganisms that are the subject of the present invention are able to produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms may be representatives of coryneform bacteria, in particular of the genus Corynebacterium. In the genus Corynebacterium there should in particular be mentioned the species Corynebacterium glutamicum, which is known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum (C. glutamicum), are in particular the known wild type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains produced therefrom.

The inventors have successfully isolated from C. glutamicum the new sigC gene coding for the enzyme sigma factor C.

In order to isolate the sigC gene or also other genes from C. glutamicum, a gene library of this microorganism is first of all incorporated in Escherichia coli (E. coli). The incorporation of gene libraries is described in generally known textbooks and manuals. As examples there may be mentioned the textbook by Winnacker: Gene and Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A very well-known gene library is that of the E. coli K-12 strain W3110, which was incorporated by Kohara et al. (Cell 50, 495-508 (1987)) into λ vectors. Bathe et al. (Molecular and general genetics, 252:255-265, 1996) describe a gene library of C. glutamicum ATCC13032 that has been incorporated by means of

the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)).

In order to produce a gene library of C. glutamicum in E. coli, there may also be used plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are in particular those E. coli strains that are restriction-defective and recombinant-defective. An example of such is the strain DH5αmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn then be subcloned into common vectors suitable for the sequencing and subsequently sequenced, as is described for example by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The DNA sequences obtained can then be investigated using known algorithms or sequence analysis programs, such as for example that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of C. glutamicum coding for the sigC gene was obtained in this way, and as SEQ ID No. 1 is part of the present invention. The amino acid sequence of the corresponding protein was also derived from the existing DNA sequence using the aforedescribed methods. The resultant amino acid sequence of the sigC gene product is shown in SEQ ID No. 2.

Coding DNA sequences that result from SEQ ID No. 1 due to the degeneracy of the genetic code are likewise covered by the present invention. Similarly, DNA sequences that hybridise with SEQ ID No. 1 or parts of SEQ ID No. 1 are also part of the invention. In the specialist field conservative amino acid replacements, such as for example the replacement of glycine by alanine or of aspartic acid by glutamic acid, in proteins are furthermore known as sense mutations that do not lead to any basic change in the activity of the protein, i.e. are functionally neutral. It is furthermore known that changes at the N-end and/or C-end of a protein do not significantly impair their function or indeed may even stabilise their function. The person skilled in the art can find relevant information on this in, *inter alia*, Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks and manuals on genetics and molecular biology. Amino acid sequences that are obtained in a corresponding manner from SEQ ID No. 2 are likewise covered by the invention.

In the same way, DNA sequences that hybridise with SEQ ID No. 1 or parts of SEQ ID No. 1 are also covered by the invention. Finally, DNA sequences that are produced by the

polymerase chain reaction (PCR) using primers resulting from SEQ ID No. 1, are also part of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

The person skilled in the art can find information on the identification of DNA sequences by means of hybridisation in, *inter alia*, the manual "The DIG System User's Guide for Filter Hybridization" published by Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The hybridisation takes place under strict conditions, in other words only hybrids are formed in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical. It is known that the strictness of the hybridisation conditions including the washing step is influenced or determined by varying the buffer composition, temperature and the salt concentration. The hybridisation reaction is preferably carried out under conditions that are relatively less strict compared to the wash steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

For the hybridisation reaction there may for example be used a 5x SSC buffer at a temperature of ca. 50 - 68°C. In this connection probes can also hybridise with polynucleotides that are less than 70% identical to the probe sequence. Such hybrids are less stable and are removed by washing under stringent conditions. This may be achieved for example by reducing the salt concentration to 2x SSC and then if necessary to 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), a temperature of ca. 50 - 68°C

being established. It is also possible to reduce the salt concentration down to 0.1x SSC. By stepwise raising of the hybridisation temperature in steps of ca. 1 - 2°C from 50 to 68°C, polynucleotide fragments can be isolated that are for example at least 70% or at least 80% or even at least 90% to 95% identical to the sequence of the probe that is used. Further details relating to hybridisation may be obtained in the form of so-called kits available on the market (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1603558).

The person skilled in the art can find details on the amplification of DNA sequences by means of the polymerase chain reaction (PCR) in, *inter alia*, the manual by Gait: Oligonucleotides Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

In the course of work carried in connection with the present invention it was established that coryneform bacteria after overexpression of the sigC gene produce amino acids in an improved manner.

In order to achieve an overexpression the number of copies of the corresponding genes can be increased, or alternatively the promoter and regulation region or the ribosome binding site located upstream of the structure gene can be mutated. Expression cassettes that are incorporated upstream of the structure gene act in the same way. By means of inducible promoters it is in addition possible to increase the expression in the course of the enzymatic amino acid production. The expression is similarly improved by measures aimed at prolonging the lifetime of the m-RNA. Furthermore, the enzyme activity is

also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs may either be present in plasmids having different numbers of copies, or may be integrated and amplified in the chromosome. Alternatively, an overexpression of the relevant genes may furthermore be achieved by altering the composition of the media and the culture conditions.

The person skilled in the art can find details on the above in, *inter alia*, Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), in Japanese laid open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks on genetics and molecular biology.

For the enhancement the sigC gene according to the invention was overexpressed for example by means of episomal plasmids. Suitable plasmids are those that are replicated in coryneform bacteria. Numerous known plasmid vectors, such as for example pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors,

such as for example those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891) may be used in a similar way.

Furthermore, also suitable are those plasmid vectors with the aid of which the process of gene amplification by integration in the chromosome can be employed, such as has been described for example by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for the duplication and amplification of the hom-thrB operon. In this method the complete gene is cloned into a plasmid vector that can replicate in a host (typically E. coli) but not in C. glutamicum. Suitable vectors are for example pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector that contains the gene to be amplified is then transferred by conjugation or transformation into the desired strain of C. glutamicum. The method of conjugation is described for example in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Transformation methods are described for example in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous

recombination by means of a crossover event, the resulting strain contains at least two copies of the relevant gene.

In addition it may be advantageous for the production of L-amino acids to enhance, in particular to overexpress, in addition to the sigC gene also one or more enzymes of the respective biosynthesis pathway, glycolysis, anaplerosis, citric acid cycle, pentose phosphate cycle, amino acid export and optionally regulatory proteins.

Thus for example, for the production of L-amino acids, in addition to the enhancement of the sigC gene one or more genes selected from the following group may be enhanced, in particular overexpressed:

- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene tpi coding for triosephosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene pgk coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene zwf coding for glucose-6-phosphate dehydrogenase (JP-A-09224661),
- the gene pyc coding for pyruvate carboxylase (DE-A-198 31 609),

- the gene mqo coding for malate-quinone-oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),

- the gene lysC coding for a feedback-resistant aspartate kinase (Accession No.P26512; EP-B-0387527; EP-A-0699759),

- the gene lysE coding for lysine export (DE-A-195 48 222),

- the gene hom coding for homoserine dehydrogenase (EP-A 0131171),

- the gene ilvA coding for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or the allele ilvA(Fbr) coding for a feedback-resistant threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842),

- the gene ilvBN coding for acetohydroxy acid synthase (EP-B 0356739),

- the gene ilvD coding for dihydroxy acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979),

- the gene zwal coding for the Zwa1 protein (DE: 19959328.0, DSM 13115).

Furthermore, it may be advantageous for the production of L-amino acids, in addition to the enhancement of the sigC genes also to attenuate, in particular to reduce, the expression of one or more genes selected from the group

- the gene pck coding for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),

- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478; DSM 12969),

- the gene poxB coding for pyruvate oxidase (DE: 1995 1975.7; DSM 13114),

- the gene zwa2 coding for the Zwa2 protein (DE: 19959327.2, DSM 13113).

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by for example using a weak promoter or a gene or allele that codes for a corresponding enzyme having a low activity and/or that inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

By means of these attenuation measures the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein and/or the activity or concentration of the protein in the starting microorganism.

In addition it may be advantageous for the production of amino acids, in addition to the overexpression of the sigC gene also to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention are likewise the subject of the invention and may be cultivated

continuously or batchwise in a batch process (batch cultivation) or in a fed batch process (feed process) or repeated fed batch process (repetitive feed process) for the purposes of production of amino acids. A summary of know cultivation methods is given in the textbook by Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must suitably satisfy the requirements of the relevant strains. Descriptions of culture media for various microorganisms are given in the manual "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Carbon sources that may be used included sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as for example soya bean oil, sunflower oil, peanut oil and coconut oil, fatty acids such as for example palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid. These substances may be used individually or as a mixture.

Nitrogen sources that may be used include organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or as a mixture.

Phosphorus sources that may be used include phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium salts. The culture medium must furthermore contain salts of metals, such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Suitable precursors may furthermore be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single one-off batch, or may be suitably metered in during the culture process.

Basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid, are used in a suitable manner in order to control the pH of the culture. Anti-foaming agents such as for example fatty acid polyglycol esters may be used to control foam formation. In order to maintain the stability of plasmids suitable selectively acting substances such as for example antibiotics may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as for example air are introduced into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until a maximum of the desired product has been formed. This objective is normally achieved within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known to the person skilled in the art. The analysis may be carried out for example as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by ion exchange chromatography

followed by ninhydrin derivatisation, or can be carried out by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention serves for the enzymatic production of amino acids.

The following microorganisms were filed as a pure culture at the German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) according to the Budapest Convention:

- Escherichia coli DH5αmcr/pEC-XK99EsigCb2ex as DSM 14375 on 29 June 2001
- Corynebacterium glutamicum DSM 5715/pEC-XK99E as DMS 13455 on 17 April 2000.

The present invention is described in more detail hereinafter with the aid of examples of implementation.

The isolation of plasmid DNA from Escherichia coli as well as all techniques involved in restriction, Klenow treatment and alkaline phosphatase treatment have been carried out by Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY, USA). Methods for the transformation of Escherichia coli are also described in this manual. The composition of readily available nutrient media such as LB or TY media are also given in the manual by Sambrook et al.

Example 1

Production of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Code no. 27-0913-02). The DNA fragments were desphosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this way was mixed with the treated ATCC13032-DNA and the batch was treated with T4-DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DN ligase, Code no. 27-0870-04). The ligation mixture was then packed into phages using the Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, Code no. 200217).

For the infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. Infection and titration of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells having been plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. Recombinant individual clones were selected after incubation overnight at 37°C.

Example 2

Isolation and sequencing of the sigC gene

The cosmid DNA of an individual colony was isolated using the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) according to the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250). After gel electrophoresis separation, the cosmid fragments were isolated in an order of magnitude of 1500 to 2000 bp using the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI,

Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture having been incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out onto LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clone was performed with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out according to the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) as modified by Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" of PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The gel electrophoresis separation and analysis of the sequencing reaction was carried out in a "rotiphoresis NF acrylamide/bisacrylamide" gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) using the "ABI Prism 377" sequencing apparatus from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequencing data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) Version 97-0. The individual sequences of the pZero1 derivates were assembled into a coherent contig. The computer-assisted coding region analysis was prepared

using the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The nucleotide sequence obtained is shown in SEQ ID No. 1. The analysis of the nucleotide sequence revealed an open reading frame of 582 base pairs, which was termed the sigC gene. The sigC gene codes for a protein of 193 amino acids.

Example 3

Production of the shuttle expression vector pEC-XK99EsigCb2ex for the enhancement of the sigC gene in C. glutamicum.

3.1 Cloning of the sigC gene

Chromosomal DNA was isolated from the strain ATCC 13032 according to the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)). The following oligonucleotides for the polymerase chain reaction were selected on the basis of the sequence of the sigC gene known from Example 2 for C. glutamicum (see SEQ ID No. 3 and SEQ ID No. 4):

sigCex1:
5` ac ggt acc-ccc tac aca cct tta tgg tg 3`
sigCex2:
5` gc tct aga-gtt gac gta gct cat ctg ct 3`

The illustrated primers were synthesised by MWG-Biotech AG (Ebersberg, Germany) and the PCR reaction was carried out according to the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) using Pwo polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction the primers permit the amplification of a

667 bp long DNA fragment that carries the sigC gene. Also, the primer sigCex1 carries the sequence for the cleavage site of the restriction endonuclease KpnI, and the primer sigCex2 contains the cleavage site of the restriction endonuclease XbaI, which are underlined in the nucleotide sequnce illustrated above.

The 667 bp long sigC fragment was cleaved with the restriction endonucleases KpnI and XbaI and then isolated from the agarose gel using the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

3.2 Construction of the shuttle vector pEC-XK99E

The E. coli - C. glutamicum shuttle vector pEC-XK99E was constructed according to the prior art. The vector contains the replication region rep of the plasmid pGA1 including the replication effector per (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the kanamycin resistance gene aph(3´)-IIa from Escherichia coli (Beck et al. (1982), Gene 19: 327-336), the replication origin, the trc promoter, the termination regions T1 and T2, the $lacI^q$ gene (repressor of the lac-operon of E.coli) and a multiple cloning site (mcs) (Norrander, J.M. et al. Gene 26, 101-106 (1983)) of the plasmid pTRC99A (Amann et al. (1988), Gene 69: 301-315).

The trc promoter can be induced by adding the lactose derivative IPTG (isopropyl-β-D-thiogalactopyranoside).

The constructed E. coli - C. glutamicum shuttle vector pEC-XK99E was transferred by means of electroporation (Liebl et al., 1989, FEMS Microbiology Letters, 53:299-303) into C. glutamicum DSM5715. The selection of the transformants was carried out on LBHIS agar consisting of 18.5 g/l brain-

heart infusion broth, 0.5 M sorbitol, 5 g/l bacto-tryptone, 2.5 g/l bacto-yeast extract, 5 g/l NaCl and 18 g/l bacto-agar that had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant by the usual methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927), cleaved with the restriction endonuclease HindIII, and the plasmid was checked by subsequent agarose gel electrophoresis.

The plasmid construct thereby obtained was termed pEC-XK99E (Fig. 1). The strain obtained by electroporation of the plasmid pEC-XK99E into the C. glutamicum strain DSM5715 was identified as DSM5715/pEC-XK99E and filed as DSM13455 in the German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) according to the Budapest Convention.

3.3 Cloning of sigC in the E. coli-C. glutamicum shuttle vector pEC-XK99E

The E. coli - C. glutamicum shuttle vector pEC-XK99E described in Example 3.2 was used as vector. DNA of this plasmid was completely cleaved with the restriction enzymes KpnI and XbaI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250).

The ca. 650 bp long sigC fragment described in Example 3.1, which was obtained by PCR and cleaved with the restriction endonucleases KpnI and XbaI, was mixed with the prepared vector pEC-XK99E and the batch was treated with T4-DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DNA ligase, Code no. 27-0870-04). The

ligation batch was transformed into the E. coli strain DH5αmcr (Hanahan, In: DNA cloning. A practical approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). The selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C recombinant individual clones were selected. Plasmid DNA was isolated from a transformant using the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) according to the manufacturer's instructions and cleaved with the restriction enzymes XbaI and KpnI in order to check the plasmid by subsequent agarose gel electrophoresis. The plasmid obtained was named pEC-XK99EsigCb2ex, and is shown in Fig. 2.

Example 4

Transformation of the strain DSM5715 with the plasmid pEC-XK99EsigCb2ex

The strain DSM5715 was transformed with the plasmid pEC-XK99EsigCb2ex using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). The selection of the transformants was carried out on LBHIS agar consisting of 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l bacto-tryptone, 2.5 g/l bacto-yeast extract, 5 g/l NaCl and 18 g/l bacto-agar that had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant by the usual methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927), cleaved with the restriction endonucleases XbaI and KpnI, and the plasmid was checked by subsequent agarose

gel electrophoresis. The strain obtained was named DSM5715/pEC-XK99EsigCb2ex1.

Example 5

Production of lysine

The C. glutamicum strain DSM5715/pEC-XK99EsigCb2ex obtained in Example 4 was cultivated in a nutrient medium suitable for the production of lysine and the lysine concentration in the culture supernatant was determined.

For this purpose the strain was first of all incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l)) for 24 hours at 33°C. A pre-culture was inoculated starting from this agar plate culture (10 ml medium in a 100 ml Erlenmeyer flask). The full medium CgIII was used as medium for the pre-culture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast Extract	10 g/l
Glucose (separately autoclaved)	2% (w/v)

The pH value was adjusted to pH 7.4

Kanamycin (25 mg/l) was added to the medium. The pre-culture was incubated for 16 hours at 33°C and at 240 rpm on a shaker mixer. A main culture was inoculated from this pre-culture so that the initial OD (660 nm) of the main culture was 0.1. The medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (separately autoclaved)	50 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 \cdot 7\ H_2O$	1.0 g/l
$CaCl_2 \cdot 2\ H_2O$	10 mg/l
$FeSO_4 \cdot 7\ H_2O$	10 mg/l
$MnSO_4 \cdot H_2O$	5.0 mg/l
Biotin (sterile filtered)	0.3 mg/l
Thiamine · HCl (sterile filtered)	0.2 mg/l
L-leucine (sterile filtered)	0.1 g/l
$CaCO_3$	25 g/l

CSL, MOPS and the salt solution were adjusted to pH 7 with ammonia water and autoclaved. The sterile substrate and vitamin solutions as well as the dry autoclaved $CaCO_3$ were then added.

The cultivation was carried out in 10 ml volume batches in a 100 ml Erlenmeyer flask equipped with baffles. Kanamycin (25 mg/l) and IPTG (1mM/l) were added. The cultivation was carried out at 33°C and 80% atmospheric humidity.

After 48 hours the OD was measured at a measurement wavelength of 660 nm using a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined using an amino acid analyser from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatisation with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	Lysine-HCl g/l
DSM5715	11.8	12.99
DSM5715/pEC-XK99EsigCb2ex	12.8	13.96

Brief description of the Figures:

Fig. 1: Map of the plasmid pEC-XK99E

Fig. 2: Map of the plasmid pEC-XK99EsigCb2ex

The abbreviations and acronyms used have the following meanings:

Kan: Kanamycin resistence gene aph(3')-IIa from Escherichia coli

HindIII	Cleavage site of the restriction enzyme HindIII
XbaI	Cleavage site of the restriction enzyme XbaI
KpnI	Cleavage site of the restriction enzyme KpnI
Ptrc	trc promoter
T1	Termination region T1
T2	Termination region T2
Per	Replication effector per
Rep	Replication region rep of the plasmid pGA1
LacIq	lacIq repressor of the lac operon of Escherichia coli
SigC	Cloned sigC gene

SEQUENCING PROTOCOL

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Thr Gln Asp Asp Val Trp Arg Leu Leu Ala His Leu Gly Gly His Glu
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Ile Ala Asp Asp Leu Thr Gln Glu Thr Tyr Leu Arg Val Met Ser Ala
    50                  55                  60

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Leu Pro Arg Phe Ala Ala Arg Ser Ser Ala Arg Thr Trp Leu Leu Ser
65                  70                  75                  80

cta gcc cgg cgc gtc tgg gtc gac aac atc cga cac gac atg gca cgc   587
Leu Ala Arg Arg Val Trp Val Asp Asn Ile Arg His Asp Met Ala Arg
                85                  90                  95

ccc cgc aaa tcc atc gtc gaa tac gaa gac acc ggt gcc acc gac gcg   635
Pro Arg Lys Ser Ile Val Glu Tyr Glu Asp Thr Gly Ala Thr Asp Ala
            100                 105                 110

agc aac gca ggc atc tgg tcc gag tgg atc gac gtg cgc acg ctt atc   683
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        115                 120                 125

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Leu Gly Tyr Thr Tyr Glu Glu Ala Ala Lys Ile Ala Asp Val Arg Val
145                 150                 155                 160

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35 40 45

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Leu Pro Arg Phe Ala Ala Arg Ser Ser Ala Arg Thr Trp Leu Leu Ser
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Gly Thr Ile Arg Ser Arg Val Ala Arg Ala Arg Ala Asp Leu Ile Ala
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Patent Claims

1. An isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence coding for the sigC gene, selected from the group

   a) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide that contains the amino acid sequence of SEQ ID No. 2,

   b) polynucleotide coding for a polypeptide that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

   c) polynucleotide that is complementary to the polynucleotides of a) or b), and

   d) polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

   the polypeptide preferably having the activity of the sigma factor C.

2. The polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA replicable in coryneform bacteria.

3. The polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.

4. The polynucleotide as claimed in claim 2, containing the nucleic acid sequence as shown in SEQ ID No. 1.

5. A replicable DNA as claimed in claim 2, containing

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence that corresponds to the sequence (i) within the region of degeneracy of the genetic code, or

(iii) at least one sequence that hybridises with the sequence that is complementary to the sequence (i) or (ii), and optionally

(iv) functionally neutral sense mutations in (i).

6. The replicable DNA as claimed in claim 5, wherein the hybridisation of sequence (iii) is carried out under conditions of stringency corresponding at most to 2x SSC.

7. The polynucleotide sequence as claimed in claim 2, that codes for a polypeptide that contains the amino acid sequence shown in SEQ ID No. 2.

8. Coryneform bacteria, in which the sigC gene is enhanced, in particular is overexpressed.

9. Escherichia coli strain DH5αmcr/pEC-XK99EsigCb2ex filed as DSM 14375 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany).

10. Corynebacterium glutamicum strain DSM5715/pEC-XK99E filed as DSM 13455 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany).

11. A process for the enzymatic production of L-amino acids, in particular lysine, wherein the following steps are carried out:

a) fermentation of the coryneform bacteria producing the desired L-amino acid, in which at least the sigC gene or nucleotide sequences coding for the latter are enhanced, in particular are overexpressed;

b) enrichment of the L-amino acid in the medium or in the cells of the bacteria, and

c) isolation of the L-amino acid.

12. The process as claimed in claim 11, wherein bacteria are used in which in addition further genes of the biosynthesis pathway of the desired L-amino acid are enhanced.

13. The process as claimed in claim 11, wherein bacteria are used in which the metabolic pathways that reduce the formation of the desired L-amino acid are at least partially switched off.

14. The process as claimed in claim 11, wherein a strain transformed with a plasmid vector is used, and the plasmid vector carries the nucleotide sequence coding for the sigC gene.

15. The process as claimed in claim 11, wherein the expression of the polynucleotide(s) that code(s) for the sigC gene is enhanced, in particular is overexpressed.

16. The process as claimed in claim 11, wherein the regulatory properties of the polypeptide (enzyme protein) for which the polynucleotide sigC codes are raised.

17. The process as claimed in claim 11, wherein for the production of L-amino acids coryneform microorganisms are fermented, in which at the same time one or more of the genes selected from the following group is enhanced or overexpressed:

17.1 the gene dapA coding for dihydrodipicolinate synthase,

17.2 the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase,

17.3 the gene tpi coding for triosephosphate isomerase,

17.4 the gene pgk coding for 3-phosphoglycerate kinase,

17.5 the gene zwf coding for glucose-6-phosphate dehydrogenase,

17.6 the gene pyc coding for pyruvate carboxylase,

17.7 the gene mqo coding for malate-quinone-oxidoreductase,

17.8 the gene lysC coding for a feedback-resistant aspartate kinase,

17.9 the gene lysE coding for lysine export,

17.10 the gene hom coding for homoserine dehydrogenase,

17.11 the gene ilvA coding for threonine dehydratase or the allele ilvA(Fbr) coding for a feedback-resistant threonine dehydratase,

17.12 the gene ilvBN coding for acetohydroxy acid synthase,

17.13 the gene ilvD coding for dihydroxy acid dehydratase,

17.14 the gene zwal coding for the Zwa1 protein.

18. The process as claimed in claim 11, wherein for the production of L-amino acids coryneform microorganisms are fermented in which at the same time one or more of the genes selected from the following group is/are attenuated:

18.1 the gene pck coding for phosphoenol pyruvate carboxykinase,

18.2 the gene pgi coding for glucose-6-phosphate isomerase,

18.3 the gene poxB coding for pyruvate oxidase,

18.4 the gene zwa2 coding for the Zwa2 protein.

19. Coryneform bacteria containing a vector that carries a polynucleotide as claimed in claim 1.

20. The process as claimed in one or more of claims 11 to 18, wherein microorganisms of the genus Corynebacterium are used.

21. The process as claimed in claim 20, wherein the Corynebacterium glutamicum strain DH5αmcr/pEC-XK99EsigCb2ex is used.

22. The process as claimed in claim 20, wherein the Corynebacterium glutamicum strain DSM5715/pEC-XK99E is used.

23. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids or polynucleotides or genes that code for the sigma factor C or that have a high degree of similarity to the sequence of the sigC gene, wherein the polynucleotide containing the polynucleotide sequences as claimed in claims 1, 2, 3 or 4 is used as hybridisation probes.

24. The process as claimed in claim 23, wherein arrays, micro arrays or DNA chips are used.

Abstract

The invention relates to an isolated polynucleotide containing a polynucleotide sequence selected from the group

a) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide that contains the amino acid sequence of SEQ ID No. 2,

b) polynucleotide coding for a polypeptide that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide that is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and a process for the enzymatic production of L-amino acids using coryneform bacteria in which at least the sigC gene is present in enhanced form, and the use of polynucleotides that contain the sequences according to the invention as hybridisation probes.

Fig. 1: Map of the plasmid pEC-XK99E

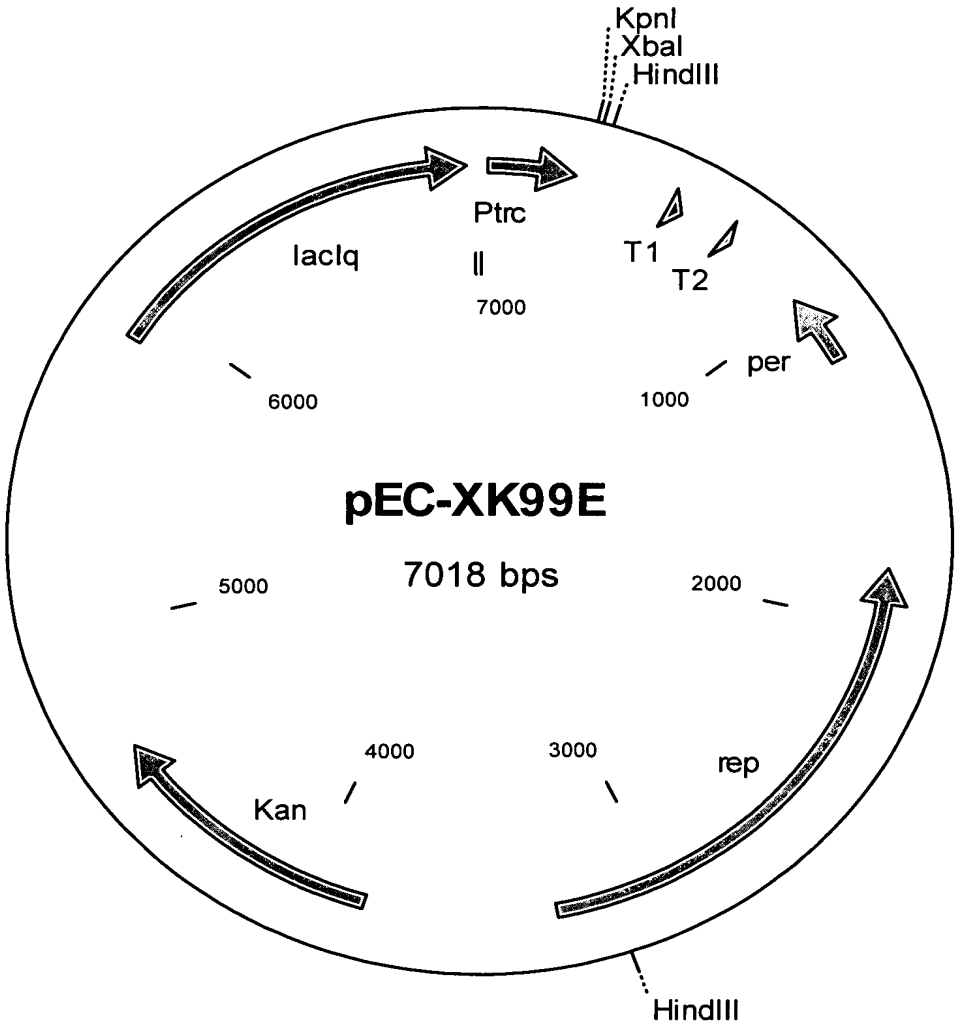
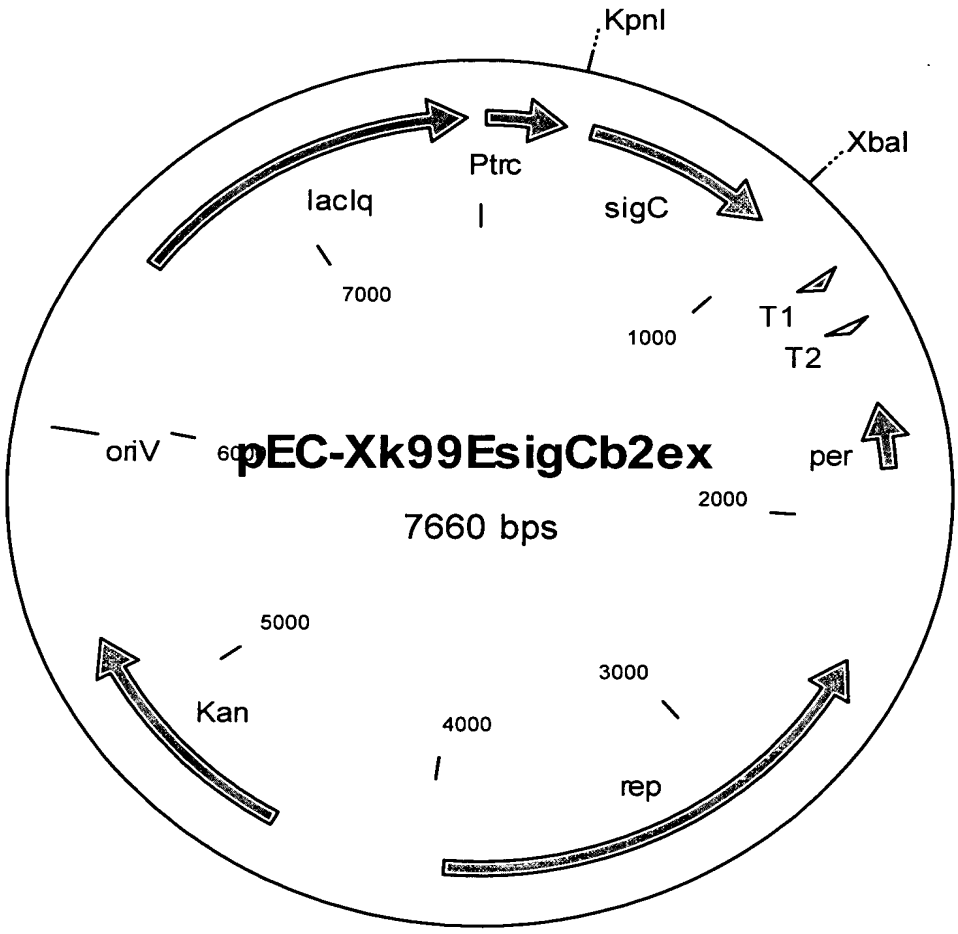


Fig. 2: Plasmid pEC-XK99EsigCb2ex



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION

I, John F. Moloney, BSc., MIL., CChem., MRSC., translator to Taylor and Meyer of 20 Kingsmead Road, London SW2 3JD, England, do solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and German languages;
2. That the following is a true translation made by me into the English language of German Priority Text Application No. 100 43 332.4;
3. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true;
   and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

Signed, this 23rd day of October 2003,

J. F. Moloney

Eynsham, Oxon OX29 4QD, England

FEDERAL REPUBLIC OF GERMANY

Certificate of Priority for Filing of a Patent Application

Filing number:	100 43 332.4
Filing date:	2nd September 2000
Applicant/Proprietor:	Degussa AG, Düsseldorf/Germany First applicant: DEGUSSA-HÜLS AKTIENGESELL-SCHAFT, Frankfurt am Main/Germany
Title:	New nucleotide sequences coding for the sigC gene
IPC:	C 12 N, C 12 Q, C 07 H

The attached papers are a true and accurate reproduction of the original documents for this patent application.

Munich, 21st June 2001

On behalf of the President of the German Patent and Trade Mark Office

(signature)

Faust

New Nucleotide Sequences Coding for the sigC gene

The subject of the present invention are nucleotide sequences of coryneform bacteria coding for the sigC gene and a process for the enzymatic production of amino acids using bacteria in which the sigC gene is enhanced.

Prior Art

L-amino acids are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry and, most especially, in animal nutrition.

It is known that amino acids can be produced by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. On account of the great importance of amino acids efforts are constantly being made to improve the production processes. Process improvements may involve fermentation technology measures such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form by for example ion exchange chromatography or the intrinsic performance properties of the microorganism itself.

In order to improve the performance properties of these microorganisms methods involving mutagenesis, selection and mutant selection are employed. In this way strains are obtained that are resistant to antimetabolites or are auxotrophic for regulatorily important metabolites, and that produce amino acids.

For some years methods of recombinant DNA technology have also been used to improve L-amino acid-producing strains of corynebacterium, by amplifying individual amino acid biosynthesis genes and investigating the effect on amino acid production.

Object of the Invention

The inventors have been involved in providing new techniques for the improved enzymatic production of amino acids.

Description of the Invention

When L-amino acids or amino acids are mentioned hereinafter, it is understood that this refers to one or more amino acids including their salts, selected from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. Lysine is particularly preferred.

The present invention provides an isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence coding for the sigC gene, selected from the group

a) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide that contains the amino acid sequence of SEQ ID No. 2,

b) polynucleotide coding for a polypeptide that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide that is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of the sigma factor C.

The present invention also provides the aforementioned polynucleotide, which is preferably a replicable DNA containing:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence that corresponds to the sequence (i) within the region of degeneracy of the genetic code, or

(iii) at least one sequence that hybridises with the sequence that is complementary to the sequence (i) or (ii), and optionally

(iv) functionally neutral sense mutations in (i).

The invention furthermore provides

a replicable polynucleotide, in particular DNA, containing the nucleotide sequence as shown in SEQ ID No. 1;

a polynucleotide coding for a polypeptide that contains the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

coryneform bacteria that contain the vector or in which the sigC gene is enhanced.

The present invention moreover provides polynucleotides that consist substantially of a polynucleotide sequence that can be obtained by screening by means of hybridisation of a corresponding gene library of a coryneform bacterium that contains the complete gene or parts thereof, with a probe that contains the sequence of the polynucleotide of the invention according to SEQ ID No. 1 or a fragment thereof, and isolation of the aforementioned polynucleotide sequence.

Polynucleotides that contain the sequences according to the invention are suitable as hybridisation probes for RNA, cDNA and DNA in order to isolate nucleic acids or polynucleotides or genes in their full length that code for the sigma factor C, or to isolate such nucleic acids or polynucleotides or genes that have a high sequence similarity to that of the sigC genes.

Polynucleotides that contain the sequences according to the invention are furthermore suitable as primers with the aid of which, and by employing the polymerase chain reaction (PCR), DNA of genes can be produced that code for the sigma factor C.

Such oligonucleotides serving as probes or primers contain at least 30, preferably at least 20, and most particularly preferably at least 15 successive nucleotides. Also suitable are oligonucleotides with a length of at least 40 or 50 nucleotides.

"Isolated" denotes separated from its natural environment.

"Polynucleotide" refers in general to polyribonucleotides and polydeoxyribonucleotides, which may be unmodified RNA or DNA or modified RNA or DNA.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment produced therefrom, and also polynucleotides that are at least 70%, preferably at least 80% and particularly preferably at least 90% to 95% identical to the polynucleotide according to SEQ ID No. 1 or a fragment produced therefrom.

The term "polypeptides" is understood to mean peptides or proteins that contain two or more amino acids bound by peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of the sigma factor C and also those that are at least 70%, preferably at least 80% and particularly preferably at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and that have the aforementioned activity.

The invention furthermore provides a process for the enzymatic production of amino acids selected from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine, using coryneform bacteria that in particular already produce amino acids and in which the nucleotide sequences coding for the sigC gene are enhanced, in particular overexpressed.

The term "enhancement" describes in this connection the raising of the intracellular activity of one or more enzymes in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, using a strong promoter, or using a gene that codes for a corresponding enzyme having a high activity, and optionally combining these measures.

The microorganisms that are the subject of the present invention are able to produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms may be representatives of coryneform bacteria, in particular of the genus Corynebacterium. In the genus Corynebacterium there should in particular be mentioned the species Corynebacterium glutamicum, which is known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum (C. glutamicum), are in particular the known wild type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains produced therefrom.

The inventors have successfully isolated from C. glutamicum the new sigC gene coding for the enzyme sigma factor C.

In order to isolate the sigC gene or also other genes from C. glutamicum, a gene library of this microorganism is first of all incorporated in Escherichia coli (E. coli). The incorporation of gene libraries is described in generally known textbooks and manuals. As examples there may be mentioned the textbook by Winnacker: Gene and Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A very well-known gene library is that of the E. coli K-12 strain W3110, which was incorporated by Kohara et al. (Cell 50, 495-508 (1987)) into λ vectors. Bathe et al. (Molecular and general genetics, 252:255-265, 1996) describe a gene library of C. glutamicum ATCC13032 that has been incorporated by means of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)).

In order to produce a gene library of C. glutamicum in E. coli, there may also be used plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are in particular those E. coli strains that are restriction-defective and recombinant-defective. An example of such is the strain DH5αmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn then be subcloned into common vectors suitable for the sequencing and subsequently sequenced, as is described for example by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The DNA sequences obtained can then be investigated using known algorithms or sequence analysis programs, such as for example that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of C. glutamicum coding for the sigC gene was obtained in this way, and as SEQ ID No. 1 is part of the present invention. The amino acid sequence of the corresponding protein was also derived from the existing DNA sequence using the aforedescribed methods. The resultant amino acid sequence of the sigC gene product is shown in SEQ ID No. 2.

Coding DNA sequences that result from SEQ ID No. 1 due to the degeneracy of the genetic code are likewise covered by

the present invention. Similarly, DNA sequences that hybridise with SEQ ID No. 1 or parts of SEQ ID No. 1 are also part of the invention. In the specialist field conservative amino acid replacements, such as for example the replacement of glycine by alanine or of aspartic acid by glutamic acid, in proteins are furthermore known as sense mutations that do not lead to any basic change in the activity of the protein, i.e. are functionally neutral. It is furthermore known that changes at the N-end and/or C-end of a protein do not significantly impair their function or indeed may even stabilise their function. The person skilled in the art can find relevant information on this in, *inter alia*, Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks and manuals on genetics and molecular biology. Amino acid sequences that are obtained in a corresponding manner from SEQ ID No. 2 are likewise covered by the invention.

In the same way, DNA sequences that hybridise with SEQ ID No. 1 or parts of SEQ ID No. 1 are also covered by the invention. Finally, DNA sequences that are produced by the polymerase chain reaction (PCR) using primers resulting from SEQ ID No. 1, are also part of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

The person skilled in the art can find information on the identification of DNA sequences by means of hybridisation in, *inter alia*, the manual "The DIG System User's Guide for Filter Hybridization" published by Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The hybridisation takes place under strict conditions, in other words only hybrids are formed in which

the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical. It is known that the strictness of the hybridisation conditions including the washing step is influenced or determined by varying the buffer composition, temperature and the salt concentration. The hybridisation reaction is preferably carried out under conditions that are relatively less strict compared to the wash steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

For the hybridisation reaction there may for example be used a 5x SSC buffer at a temperature of ca. 50 - 68°C. In this connection probes can also hybridise with polynucleotides that are less than 70% identical to the probe sequence. Such hybrids are less stable and are removed by washing under stringent conditions. This may be achieved for example by reducing the salt concentration to 2x SSC and then if necessary to 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), a temperature of ca. 50 - 68°C being established. It is also possible to reduce the salt concentration down to 0.1x SSC. By stepwise raising of the hybridisation temperature in steps of ca. 1 - 2°C from 50 to 68°C, polynucleotide fragments can be isolated that are for example at least 70% or at least 80% or even at least 90% to 95% identical to the sequence of the probe that is used. Further details relating to hybridisation may be obtained in the form of so-called kits available on the market (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1603558).

The person skilled in the art can find details on the amplification of DNA sequences by means of the polymerase chain reaction (PCR) in, *inter alia*, the manual by Gait: Oligonucleotides Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

In the course of work carried in connection with the present invention it was established that coryneform bacteria after overexpression of the sigC gene produce amino acids in an improved manner.

In order to achieve an overexpression the number of copies of the corresponding genes can be increased, or alternatively the promoter and regulation region or the ribosome binding site located upstream of the structure gene can be mutated. Expression cassettes that are incorporated upstream of the structure gene act in the same way. By means of inducible promoters it is in addition possible to increase the expression in the course of the enzymatic amino acid production. The expression is similarly improved by measures aimed at prolonging the lifetime of the m-RNA. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs may either be present in plasmids having different numbers of copies, or may be integrated and amplified in the chromosome. Alternatively, an overexpression of the relevant genes may furthermore be achieved by altering the composition of the media and the culture conditions.

The person skilled in the art can find details on the above in, *inter alia*, Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), in Japanese laid open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and

Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks on genetics and molecular biology.

For the enhancement the sigC gene according to the invention was overexpressed for example by means of episomal plasmids. Suitable plasmids are those that are replicated in coryneform bacteria. Numerous known plasmid vectors, such as for example pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as for example those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891) may be used in a similar way.

Furthermore, also suitable are those plasmid vectors with the aid of which the process of gene amplification by integration in the chromosome can be employed, such as has been described for example by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for the duplication and amplification of the hom-thrB operon. In this method the complete gene is cloned into a plasmid vector that can replicate in a host (typically E. coli) but not in C. glutamicum. Suitable vectors are for example pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector that contains the gene to be amplified is then

transferred by conjugation or transformation into the desired strain of C. glutamicum. The method of conjugation is described for example in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Transformation methods are described for example in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a crossover event, the resulting strain contains at least two copies of the relevant gene.

In addition it may be advantageous for the production of L-amino acids to enhance, in particular to overexpress, in addition to the sigC gene also one or more enzymes of the respective biosynthesis pathway, glycolysis, anaplerosis, citric acid cycle, pentose phosphate cycle, amino acid export and optionally regulatory proteins.

Thus for example, for the production of L-amino acids, in addition to the enhancement of the sigC gene one or more genes selected from the following group may be enhanced, in particular overexpressed:

- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene tpi coding for triosephosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene pgk coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the gene zwf coding for glucose-6-phosphate dehydrogenase (JP-A-09224661),

- the gene pyc coding for pyruvate carboxylase (DE-A-198 31 609),
- the gene mqo coding for malate-quinone-oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the gene lysC coding for a feedback-resistant aspartate kinase (Accession No.P26512),
- the gene lysE coding for lysine export (DE-A-195 48 222),
- the gene hom coding for homoserine dehydrogenase (EP-A 0131171),
- the gene ilvA coding for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or the allele ilvA(Fbr) coding for a feedback-resistant threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842),
- the gene ilvBN coding for acetohydroxy acid synthase (EP-B 0356739),
- the gene ilvD coding for dihydroxy acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979),
- the gene zwal coding for the Zwal protein (DE: 19959328.0, DSM 13115).

Furthermore, it may be advantageous for the production of L-amino acids, in addition to the enhancement of the sigC genes also to attenuate, in particular to reduce, the expression of one or more genes selected from the group

- the gene pck coding for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),

- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478; DSM 12969),
- the gene poxB coding for pyruvate oxidase (DE: 1995 1975.7; DSM 13114),
- the gene zwa2 coding for the Zwa2 protein (DE: 19959327.2, DSM 13113).

In addition it may be advantageous for the production of amino acids, in addition to the overexpression of the sigC gene also to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention are likewise the subject of the invention and may be cultivated continuously or batchwise in a batch process (batch cultivation) or in a fed batch process (feed process) or repeated fed batch process (repetitive feed process) for the purposes of production of amino acids. A summary of know cultivation methods is given in the textbook by Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must suitably satisfy the requirements of the relevant strains. Descriptions of culture media for various microorganisms are given in the manual "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Carbon sources that may be used included sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose,

oils and fats such as for example soya bean oil, sunflower oil, peanut oil and coconut oil, fatty acids such as for example palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid. These substances may be used individually or as a mixture.

Nitrogen sources that may be used include organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or as a mixture.

Phosphorus sources that may be used include phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium salts. The culture medium must furthermore contain salts of metals, such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Suitable precursors may furthermore be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single one-off batch, or may be suitably metered in during the culture process.

Basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid, are used in a suitable manner in order to control the pH of the culture. Anti-foaming agents such as for example fatty acid polyglycol esters may be used to control foam formation. In order to maintain the stability of plasmids suitable selectively acting substances such as for example antibiotics may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing

gas mixtures such as for example air are introduced into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until a maximum of the desired product has been formed. This objective is normally achieved within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known to the person skilled in the art. The analysis may be carried out for example as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by ion exchange chromatography followed by ninhydrin derivatisation, or can be carried out by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention serves for the enzymatic production of amino acids.

The present invention is described in more detail hereinafter with the aid of examples of implementation.

The isolation of plasmid DNA from Escherichia coli as well as all techniques involved in restriction, Klenow treatment and alkaline phosphatase treatment have been carried out by Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY, USA). Methods for the transformation of Escherichia coli are also described in this manual.

The composition of readily available nutrient media such as LB or TY media are also given in the manual by Sambrook et al.

Example 1

Production of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Code no. 27-0913-02). The DNA fragments were desphosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this way was mixed with the treated ATCC13032-DNA and the batch was treated with T4-DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DN ligase, Code no. 27-0870-04). The ligation mixture was then packed into phages using the Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, Code no. 200217).

For the infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. Infection and titration of the cosmid library were carried out as described by Sambrook et

al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells having been plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. Recombinant individual clones were selected after incubation overnight at 37°C.

Example 2

Isolation and sequencing of the sigC gene

The cosmid DNA of an individual colony was isolated using the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) according to the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250). After gel electrophoresis separation, the cosmid fragments were isolated in an order of magnitude of 1500 to 2000 bp using the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture having been incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences

U.S.A., 87:4645-4649) and plated out onto LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clone was performed with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out according to the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) as modified by Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" of PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The gel electrophoresis separation and analysis of the sequencing reaction was carried out in a "rotiphoresis NF acrylamide/bisacrylamide" gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) using the "ABI Prism 377" sequencing apparatus from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequencing data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) Version 97-0. The individual sequences of the pZero1 derivates were assembled into a coherent contig. The computer-assisted coding region analysis was prepared using the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The nucleotide sequence obtained is shown in SEQ ID No. 1. The analysis of the nucleotide sequence revealed an open reading frame of 582 base pairs, which was termed the sigC gene. The sigC gene codes for a protein of 193 amino acids.

SEQUENCING PROTOCOL

<110> Degussa-Hüls AG

<120> New nucleotide sequences coding for the sigC gene

<130> oooooo BT

<140>
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<170> PatentIn Ver. 2.1

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ttacctatgg attaagtctg attgatagtc tacatcagaa tgtcacttcg cgccaccaaa 180

taatcagccc ttacgtaaac tgccagcaaa aagacaaaag tatgatactt tttgcccact 240

ttgacacccc ctacacacct ttatggtgac cccggtctga actggtattc tgagcaatt 299

gtg aag tca aaa gag cgt aac gac gcc cac gtc acc gag ctg gcc cta 347
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1 5 10 15

gcc gcc ggc cgt ggc gac cgc gca gct ctc acc gat ttc atc cgg gaa 395
Ala Ala Gly Arg Gly Asp Arg Ala Ala Leu Thr Asp Phe Ile Arg Glu
20 25 30

acc caa gac gat gtc tgg cgt ctc ctc gcc cac ctt ggc ggc cac gaa 443
Thr Gln Asp Asp Val Trp Arg Leu Leu Ala His Leu Gly Gly His Glu
35 40 45

atc gcc gac gat cta acc caa gaa act tat ctg cgg gtc atg agc gcc 491
Ile Ala Asp Asp Leu Thr Gln Glu Thr Tyr Leu Arg Val Met Ser Ala
50 55 60

ctc ccc cgc ttc gca gcg cgc tcc tcg gcg cgt acc tgg cta cta tcg 539
Leu Pro Arg Phe Ala Ala Arg Ser Ser Ala Arg Thr Trp Leu Leu Ser
65 70 75 80

cta gcc cgg cgc gtc tgg gtc gac aac atc cga cac gac atg gca cgc 587
Leu Ala Arg Arg Val Trp Val Asp Asn Ile Arg His Asp Met Ala Arg
85 90 95

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ccc cgc aaa tcc atc gtc gaa tac gaa gac acc ggt gcc acc gac gcg   635
Pro Arg Lys Ser Ile Val Glu Tyr Glu Asp Thr Gly Ala Thr Asp Ala
            100                 105                 110

agc aac gca ggc atc tgg tcc gag tgg atc gac gtg cgc acg ctt atc   683
Ser Asn Ala Gly Ile Trp Ser Glu Trp Ile Asp Val Arg Thr Leu Ile
        115                 120                 125

gac gcc ctc cca ccc gaa cgc cgc gaa gcc ctc atc ctc acc caa gtg   731
Asp Ala Leu Pro Pro Glu Arg Arg Glu Ala Leu Ile Leu Thr Gln Val
    130                 135                 140

ttg ggc tac acc tac gaa gaa gcc gca aaa atc gcc gac gtc cga gtc   779
Leu Gly Tyr Thr Tyr Glu Glu Ala Ala Lys Ile Ala Asp Val Arg Val
145                 150                 155                 160

gga aca atc cgt tcc cgc gta gcc cgc gcc aga gcg gac ctc att gct   827
Gly Thr Ile Arg Ser Arg Val Ala Arg Ala Arg Ala Asp Leu Ile Ala
                165                 170                 175

gca aca gct acc ggt gat tcc tca gcc gaa gat ggc aaa tcc gcc caa   875
Ala Thr Ala Thr Gly Asp Ser Ser Ala Glu Asp Gly Lys Ser Ala Gln
            180                 185                 190

ggt tagcagatga gctacgtcaa cggcgtaatc ccttaaccag attgctaatt        928
Gly

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            20                  25                  30

Thr Gln Asp Asp Val Trp Arg Leu Leu Ala His Leu Gly Gly His Glu
        35                  40                  45

Ile Ala Asp Asp Leu Thr Gln Glu Thr Tyr Leu Arg Val Met Ser Ala
    50                  55                  60

Leu Pro Arg Phe Ala Ala Arg Ser Ser Ala Arg Thr Trp Leu Leu Ser
65                  70                  75                  80

Leu Ala Arg Arg Val Trp Val Asp Asn Ile Arg His Asp Met Ala Arg
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85 90 95

Pro Arg Lys Ser Ile Val Glu Tyr Glu Asp Thr Gly Ala Thr Asp Ala
100 105 110

Ser Asn Ala Gly Ile Trp Ser Glu Trp Ile Asp Val Arg Thr Leu Ile
115 120 125

Asp Ala Leu Pro Pro Glu Arg Arg Glu Ala Leu Ile Leu Thr Gln Val
130 135 140

Leu Gly Tyr Thr Tyr Glu Glu Ala Ala Lys Ile Ala Asp Val Arg Val
145 150 155 160

Gly Thr Ile Arg Ser Arg Val Ala Arg Ala Arg Ala Asp Leu Ile Ala
165 170 175

Ala Thr Ala Thr Gly Asp Ser Ser Ala Glu Asp Gly Lys Ser Ala Gln
180 185 190

Gly

Patent Claims

1. An isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence coding for the sigC gene, selected from the group

   a) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide that contains the amino acid sequence of SEQ ID No. 2,

   b) polynucleotide coding for a polypeptide that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

   c) polynucleotide that is complementary to the polynucleotides of a) or b), and

   d) polynucleotide containing at least at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

   the polypeptide preferably having the activity of the sigma factor C.

2. The polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA replicable in coryneform bacteria.

3. The polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.

4. The polynucleotide as claimed in claim 2, containing the nucleic acid sequence as shown in SEQ ID No. 1.

5. A replicable DNA as claimed in claim 2, containing

   (i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence that corresponds to the sequence (i) within the region of degeneracy of the genetic code, or

(iii) at least one sequence that hybridises with the sequence that is complementary to the sequence(i) or (ii), and optionally

(iv) functionally neutral sense mutations in (i).

6. The replicable DNA as claimed in claim 5, wherein the hybridisation of sequence (iii) is carried out under conditions of stringency corresponding at most to 2x SSC.

7. The polynucleotide sequence as claimed in claim 2, that codes for a polypeptide that contains the amino acid sequence shown in SEQ ID No. 2.

8. Coryneform bacteria, in which the sigC gene is enhanced, in particular is overexpressed.

9. A process for the enzymatic production of L-amino acids, in particular lysine, wherein the following steps are carried out:

a) fermentation of the coryneform bacteria producing the desired L-amino acid, in which at least the sigC gene or nucleotide sequences coding for the latter are enhanced, in particular are overexpressed;

b) enrichment of the L-amino acid in the medium or in the cells of the bacteria, and

c) isolation of the L-amino acid.

10. The process as claimed in claim 9, wherein bacteria are used in which in addition further genes of the

biosynthesis pathway of the desired L-amino acid are enhanced.

11. The process as claimed in claim 9, wherein bacteria are used in which the metabolic pathways that reduce the formation of the desired L-amino acid are at least partially switched off.

12. The process as claimed in claim 9, wherein a strain transformed with a plasmid vector is used, and the plasmid vector carries the nucleotide sequence coding for the sigC gene.

13. The process as claimed in claim 9, wherein the expression of the polynucleotide(s) that codes for the sigC gene is enhanced, in particular is overexpressed.

14. The process as claimed in claim 8, wherein the regulatory properties of the polypeptide (enzyme protein) for which the polynucleotide sigC codes are raised.

15. The process as claimed in claim 9, wherein for the production of L-amino acids coryneform microorganisms are fermented, in which at the same time one or more of the genes selected from the following group is enhanced or overexpressed:

15.1 the gene dapA coding for dihydrodipicolinate synthase,

15.2 the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase,

15.3 the gene tpi coding for triosephosphate isomerase,

15.4 the gene pgk coding for 3-phosphoglycerate kinase,

15.5 the gene zwf coding for glucose-6-phosphate dehydrogenase,

15.6 the gene pyc coding for pyruvate carboxylase,

15.7 the gene mqo coding for malate-quinone-oxidoreductase,

15.8 the gene lysC coding for a feedback-resistant aspartate kinase,

15.9 the gene lysE coding for lysine export,

15.10 the gene hom coding for homoserine dehydrogenase,

15.11 the gene ilvA coding for threonine dehydratase or the allele ilvA(Fbr) coding for a feedback-resistant threonine dehydratase,

15.12 the gene ilvBN coding for acetohydroxy acid synthase,

15.13 the gene ilvD coding for dihydroxy acid dehydratase,

15.14 the gene zwal coding for the Zwa1 protein.

16. The process as claimed in claim 9, wherein for the production of L-amino acids coryneform microorganisms are fermented in which at the same time one or more of the genes selected from the following group is/are attenuated:

16.1 the gene pck coding for phosphoenol pyruvate carboxykinase,

16.2 the gene pgi coding for glucose-6-phosphate isomerase,

16.3 the gene poxB coding for pyruvate oxidase,

16.4 the gene zwa2 coding for the Zwa2 protein.

17. Coryneform bacteria containing a vector that carries a polynucleotide as claimed in claim 1.

18. The process as claimed in one or more of the preceding claims, wherein microorganisms of the genus Corynebacterium are used.

19. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids or polynucleotides or genes that code for the sigma factor C or that have a high degree of similarity to the sequence of the sigC gene, wherein the polynucleotide containing the polynucleotide sequences as claimed in claims 1, 2, 3 or 4 is used as hybridisation probes.

Abstract

The invention relates to an isolated polynucleotide containing a polynucleotide sequence selected from the group

a) polynucleotide that is at least 70% identical to a polynucleotide coding for a polypeptide that contains the amino acid sequence of SEQ ID No. 2,

b) polynucleotide coding for a polypeptide that contains an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide that is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and a process for the enzymatic production of L-amino acids using coryneform bacteria in which at least the sigC gene is present in enhanced form, and the use of polynucleotides that contain the sequences according to the invention as hybridisation probes.